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Role of Heme Oxygenase-1 in Polymyxin B-Induced Nephrotoxicity in Rats

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Polymyxin B (PMB) is a cationic polypeptide antibiotic with activity against multidrug-resistant Gram-negative bacteria. PMB-induced nephrotoxicity consists of direct toxicity to the renal tubules and the release of reactive oxygen species (ROS) with oxidative damage. This study evaluated the nephroprotective effect of heme oxygenase-1 (HO-1) against PMB-induced nephrotoxicity in rats. Adult male Wistar rats, weighing 286 ± 12 g, were treated intraperitoneally once a day for 5 days with saline, hemin (HO-1 inducer; 10 mg/kg), zinc protoporphyrin (ZnPP) (HO-1 inhibitor; 50 μmol/kg, administered before PMB on day 5), PMB (4 mg/kg), PMB plus hemin, and PMB plus ZnPP. Renal function (creatinine clearance, Jaffe method), urinary peroxides (ferrous oxidation of xylenol orange version 2 [FOX-2]), urinary thiobarbituric acid-reactive substances (TBARS), renal tissue thiols, catalase activity, and renal tissue histology were analyzed. The results showed that PMB reduced creatinine clearance ($P < 0.05$), with an increase in urinary peroxides and TBARS. The PMB toxicity caused a reduction in catalase activity and thiols ($P < 0.05$). Hemin attenuated PMB nephrotoxicity by increasing the catalase antioxidant activity ($P < 0.05$). The combination of PMB and ZnPP incremented the fractional interstitial area of renal tissue ($P < 0.05$), and acute tubular necrosis in the cortex area was also observed. This is the first study demonstrating the protective effect of HO-1 against PMB-induced nephrotoxicity.

Polymyxin B sulfate (PMB), for which early use in the 1970s was discontinued because of its toxicity, especially nephrotoxicity, has reemerged in clinical practice over the last 10 years (9, 13, 14, 22, 26, 42). The current reuse of PMB is the result of the growing number of infections caused by the highly mutational and adaptive resistance of Gram-negative bacteria (6, 9, 13, 14). The propagation of infections caused by multidrug-resistant microorganisms in seriously ill patients contributes to the increase in the number of cases of drug-induced toxic acute kidney injury (AKI) (9, 13, 14, 24, 26, 42). An epidemiological study showed that nephrotoxic drugs are contributing factors in 19 to 25% of cases of severe AKI in critically ill patients (41).

PMB induces nephrotoxicity characterized by acute tubular necrosis (ATN) and elevated serum creatinine concentration in 37% of patients (20, 25, 31). Many drugs found to cause nephrotoxicity exert toxic effects by one or more pathogenic mechanisms. These include altered glomerular hemodynamics, tubular cell toxicity, inflammation, oxidative injury, crystal nephropathy, or thrombotic microangiopathy (33, 34). Biotransformations of drugs favor the formation of toxic metabolites and reactive oxygen species (ROS). These products of metabolism tilt the balance in favor of oxidative stress, which outstrips antioxidant enzymes and increases renal injury via nucleic acid oxidation, protein damage, and DNA strand breaks (34).

Several studies in animal models suggest that oxidative stress has a key role in polymyxin family-induced nephrotoxicity (32, 46, 47). ROS has also been suggested to a play role in nephrotoxicity induced by gentamicin, cisplatin, and radiocontrast agents (26, 28). Simultaneously, some proteins (e.g., heat shock protein 32) exert a renoprotective effect against oxidative injury, and among these proteins, the cytoprotective role of heme oxygenase-1 (HO-1) is emphasized (8, 38, 39, 40).

Redox imbalance is damaging to the cell, and it also induces protection mechanisms such as the activation of heme oxygenases (3, 4). Heme oxygenases are responsible for the degradation of the heme group, an iron-porphyrin complex derived from various heme proteins such as hemoglobin, myoglobin, and mitochondrial and microsomal cytochromes (4). The heme group plays an important role in the maintenance of cellular functions, including oxygenation, respiration, and cell signaling (1, 4).

HO-1 is one of the three isoforms of heme oxygenases that is extensively studied as an effective cytoprotective system, in which antioxidant activity is the first mechanism to be considered. Induction of HO-1 increases heme degradation, reducing the formation of this potentially toxic prooxidant (3, 15, 23). Heme degradation by the heme oxygenase enzymatic system also induces the synthesis of ferritin, which acts as scavengers of free iron and thus prevents its participation in oxidative damage (36, 39).

Studies using models of radiocontrast agent-induced AKI demonstrate a significant renoprotective effect after the induction of HO-1 (18). The participation of HO-1 in the redox imbalance in models of 30-min ischemic AKI ameliorated renal function and elevated levels of thiols and catalase, accompanied by a reduction in malondialdehyde (MDA) (44). The cytoprotective effect of heme oxygenase induction is also demonstrated in toxicity of gentamicin on NRK-52E cells by reducing apoptosis and optimizing cell viability (38).

Considering the above-mentioned beneficial role of the HO-1 inducer, our study was aimed at examining its effect in the restoration of oxidant injury in the PMB nephrotoxicity.

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MATERIALS AND METHODS

Chemicals and reagents. The following chemicals and reagents were used in this study: polymyxin B, Bedford Laboratories; hemin, Fluka Chemin, Sigma-Aldrich (Switzerland); zinc protoporphyrin (ZnPP), Sigma; xylenol orange [o-cresolsulfonphthalein-3′,3″-bis(methylimino) diacetic acid], Sigma; DTPA (diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetate), Sigma; and DTNB [5,5′-dithiobis(2-nitrobenzoic acid)], Sigma.

Animals. This study was approved by the Ethical Committee of Experimental Animals, University of Sao Paulo (CEEA; registration no. 038/08), and was performed in accordance with international standards for the manipulation and care of laboratory animals. Adult male Wistar rats were housed in a room at a controlled temperature (25°C/77°F) on alternating light/dark cycles and had free access to water and rat chow (Nuvilab CR-1; Nuvital, Brazil).

Groups of treatments. Rats weighing 286 ± 12 g were divided into the following six groups: (i) saline group (control group), rats receiving 3 ml/kg 0.9% NaCl, intraperitoneally (i.p.), once a day for 5 days; (ii) hemin group, rats receiving 10 mg/kg hemin (11), i.p., once a day for 5 days; (iii) ZnPP group, rats receiving 50 μmol/kg zinc protoporphyrin (27), i.p., on day 5; (iv) PMB group, rats receiving 4 mg/kg PMB (10), i.p., once a day for 5 days; (v) PMB plus hemin group, rats receiving 4 mg/kg PMB, i.p., plus 10 mg/kg hemin, i.p., once a day for 5 days; and (vi) PMB plus ZnPP group, rats receiving 4 mg/kg PMB, i.p., once a day for 5 days, plus 50 μmol/kg ZnPP, i.p., administered before PMB on day five.

Procedures and timing. (i) Metabolic cages for collection of urine sample. On the fifth day, immediately after the last injection, rats were placed in metabolic cages for the measurement of 24-h urinary volume and the collection of a urine sample.

(ii) Collection of blood sample. On the sixth day, the animals were anesthetized with sodium thiopental (30 to 40 mg/kg; Cristália, Brazil) to collect a blood sample by puncture of the abdominal aorta. At the end of the experiment, the animals were submitted to euthanasia according to guidelines for animal experimentation.

(iii) Tissue sample collection/preparation. The right kidney was removed and immediately cooled to the temperature needed for the ROS metabolite assay. The left kidney was perfused with 100 ml phosphate-buffered saline (PBS) and 60 ml 4% paraformaldehyde and then cooled at 4°C for 2 h. After this period, the kidney was immersed in Bouin solution for 4 h at room temperature. Renal tissue was then submitted to successive baths in 70% alcohol for the elimination of picric acid, dehydrated and embedded in paraffin. The paraffin sections of perfused/fixed kidneys were stained with hematoxylin and eosin for histological analysis under light microscopy.

Creatinine clearance. Renal function was evaluated based on creatinine clearance. Serum and urinary creatinine was measured using the Jaffe method and calculated with the following formula: creatinine clearance = (urinary creatinine × 24-h urinary volume)/serum creatinine. The calculated clearance was adjusted by 100 g/rat weight (12, 44).

Determination of urinary peroxides. Urinary peroxides were determined by the ferrous oxidation of xylenol orange version 2 (FOX-2) method. Xylenol orange shows a high selectivity for the Fe^{3+} ion, producing a bluish purple complex ($A = 4.3 \times 10^4\ M^{-1}\ cm^{-1}$). The values were corrected for gram urinary creatinine and are expressed as nmol/g creatinine (19, 45).

Measurement of TBARS. The lipid peroxidation levels of malondialdehyde were determined by methods measuring thiobarbituric acid-reactive substances (TBARS) (21). For the quantification of TBARS in urine, 0.4 ml of a urine sample with 0.6 ml water was added to a reaction mixture consisting of 1.0 ml 17.5% trichloroacetic acid (TCA) and 1.0 ml 0.6% thiobarbituric acid. This mixture was heated in a water bath at 95°C for 20 min for the reaction with thiobarbituric acid. Next, the solution was removed from the water bath and cooled on ice, followed by the addition of 1.0 ml 70% TCA. The solution was homogenized and incubated for 20 min. Finally, the solution was centrifuged at 3,000 rpm for 15 min and the absorbance was read in a spectrophotometer at 534 nm. The amount of MDA was calculated using a molar extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$. Values are expressed as nmol/g creatinine (43).

TABLE 1 Body weight and overall renal function of rats treated with saline, hemin, ZnPP, PMB, PMB plus hemin, and PMB plus ZnPP[a]

Group (no. of rats)	Body weight (g)	Urinary output (ml/min)	CL_{CR}/100 g (ml/min)
Saline (6)	297 ± 05	0.0058 ± 0.0022	0.7 ± 0.1
Hemin (6)	296 ± 16	0.0084 ± 0.0018	0.8 ± 0.1
ZnPP (7)	291 ± 25	0.0064 ± 0.0044	0.5 ± 0.1
PMB (7)	264 ± 16[b]	0.0096 ± 0.0091	0.3 ± 0.3[b]
PMB plus hemin (7)	286 ± 12	0.0119 ± 0.0059	0.5 ± 0.1[b,c]
PMB plus ZnPP (6)	284 ± 18	0.0155 ± 0.0105	0.3 ± 0.1[b,d]

[a] Results are reported as means ± standard errors of the means. CL_{CR}, creatinine clearance.
[b] $P < 0.05$ versus the value for saline.
[c] $P < 0.05$ versus the value for PMB.
[d] $P < 0.05$ versus the value for PMB plus hemin.

Analysis of soluble nonprotein thiols in renal tissue. The renal tissue was homogenized in 1 ml of a solution containing 10 mM sodium acetate, 0.5% Tween 20, and 100 μM DTPA (diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetate), pH 6.5. One aliquot was reserved for the immediate measurement of total protein and the other aliquot was precipitated with 10% TCA for the measurement of total thiols. The deproteinized samples were homogenized in 300 μl of a solution containing 1 mM DTNB [5,5′-dithiobis(2-nitrobenzoic acid)] and 100 mM Tris buffer, pH 8.0. After 10 min at room temperature, the quantity of thiols was determined as the mean absorbance at 412 nm ($A = 13.6 \times 10^3\ M^{-1}\ cm^{-1}$) (5). The amount of soluble thiols was corrected for total protein and is expressed as nmol/mg total protein (35).

Analysis of catalase activity in renal tissue. Catalase activity was measured by the homogenization of renal tissue in PBS. One aliquot was used for the measurement of total protein. The other aliquot was mixed with 1 M Tris-HCl, 50 mM EDTA, and 10 mM H_2O_2, and the absorbance was read in a spectrophotometer at 240 nm for 2 min at room temperature. The results are reported as the decrease in nmol H_2O_2 per min/mg total protein (2).

Histological and morphometric analysis. Tubulointerstitial damage was defined as tubular necrosis, presence of an inflammatory cell infiltrate, tubular lumen dilatation, or tubular atrophy.

The fractional interstitial area (FIA) of the renal cortex was determined by morphometry (Axioskop 40; Carl Zeiss, Germany). Interstitial areas were first manually encircled on a video screen and then determined by computerized morphometry in different experimental groups in each renal cortex section, and 20 grid fields (0.174 mm^2 per animal) were evaluated.

Statistical analysis. One-factor analysis of variance (ANOVA) with confidence intervals for the mean and pairwise comparisons was used. Overlapping intervals indicated no difference between treatments, which was subsequently confirmed by the Tukey test. The results are reported as the mean ± the standard error of the mean. A P value of <0.05 was considered to be significant.

RESULTS

The hemin and ZnPP groups were similar (not statistically different) to the saline group in creatinine clearance and metabolites of ROS; thus, saline was referred to as the control to the PMB groups (Table 1; see also Fig. 1 to 4).

As shown in Table 1, pretreatment with PMB for 5 days resulted in a significant decrease in the creatinine clearance with the maintenance of urinary output, characterizing the model of AKI due to PMB-induced nephrotoxicity ($P < 0.05$).

Animals treated with PMB and the HO-1 inducer (hemin) pre-

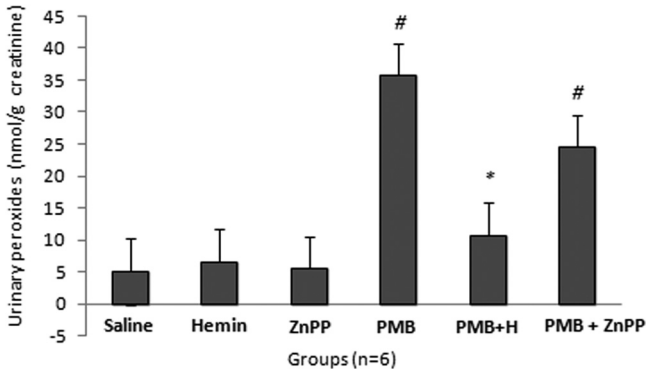

FIG 1 Mean (±standard deviation [SD]) urinary peroxides in the saline, hemin, ZnPP, PMB, PMB plus hemin, and PMB plus ZnPP groups. #, significantly different from the saline group ($P < 0.05$); *, significantly different from the PMB group ($P < 0.05$).

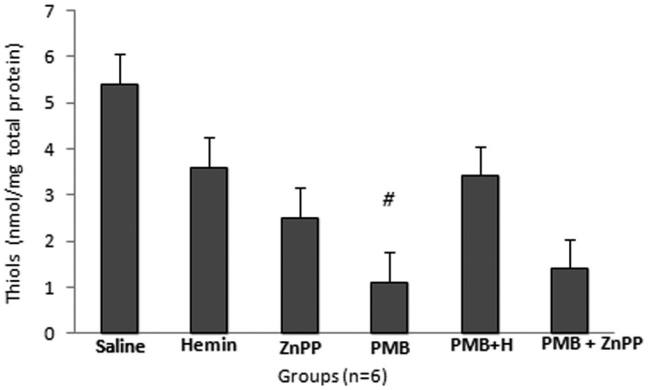

FIG 3 Mean (±SD) thiols in the saline, hemin, ZnPP, PMB, PMB plus hemin, and PMB plus ZnPP groups. #, significantly different from the saline group ($P < 0.05$).

sented significant attenuation of PMB-induced renal dysfunction compared to the PMB group ($P < 0.05$). In contrast, a reduction in creatinine clearance was observed in the PMB group receiving the HO-1 inhibitor (ZnPP) compared to the level in the PMB plus hemin group ($P < 0.05$), with no difference from that in the PMB group.

The PMB group presented a significant increase in urinary peroxide levels (Fig. 1) compared to the saline control (35.6 ± 9.9 nmol/g creatinine versus 5.0 ± 1.3 nmol/g creatinine). A reduction in urinary peroxide excretion was observed in the PMB group pretreated with hemin compared to the PMB group (10.7 ± 2.0 nmol/g creatinine versus 35.6 ± 9.9 nmol/g creatinine). Nevertheless, this group differed from the saline group (10.7 ± 2.0 nmol/g creatinine versus 5.0 ± 1.3 nmol/g creatinine). In contrast, a nonsignificant reduction in urinary peroxides was observed in animals pretreated with the HO inhibitor (ZnPP) compared to the level in the PMB group (24.4 ± 12.5 nmol/g creatinine versus 35.6 ± 9.9 nmol/g creatinine), but this marker was higher than the PMB plus hemin group marker.

With respect to TBARS (Fig. 2), levels were higher in the PMB group than in the saline group, as expected (98.1 ± 35.7 nmol/g creatinine versus 34.4 ± 12.0 nmol/g creatinine). TBARS levels were similar in the PMB plus hemin and saline groups (46.6 ± 7.9 nmol/g creatinine; 34.4 ± 12.0 nmol/g creatinine, respectively), but a significant difference was observed between the PMB and PMB plus hemin groups (98.1 ± 35.7 nmol/g creatinine versus 46.6 ± 7.9 nmol/g creatinine). On the other hand, lower TBARS levels were also observed in the PMB group treated with the HO inhibitor (ZnPP) than those exhibited by the PMB group.

Figure 3 shows antioxidant activity; PMB toxicity caused a reduction in thiols compared to the saline group (15.7 ± 1.3 nmol/mg total protein versus 34.7 ± 10.9 nmol/mg total protein). Thiols levels were higher in the PMB hemin and PMB plus ZnPP groups than those presented by PMB (28.0 ± 11.9 nmol/mg total protein versus 21.6 ± 9.3 nmol/mg total protein versus 15.7 ± 1.3 nmol/mg total protein), but this difference was not significant.

Treatment with PMB plus hemin increased the catalase antioxidant activity (3.4 ± 1.0 nmol H_2O_2 min/mg total protein versus 1.1 ± 0.3 nmol H_2O_2 min/mg total protein) and reduced the catalase levels in the PMB plus ZnPP group compared to the saline group (1.4 ± 0.3 nmol H_2O_2 min/mg total protein versus 5.4 ± 0.9 nmol H_2O_2 min/mg total protein) (Fig. 4).

Histological analyses of the PMB (Fig. 5B) and PMB plus ZnPP (Fig. 5C) groups showed the presence of injury characterized by edema and diffuse inflammatory infiltration of the interstitium,

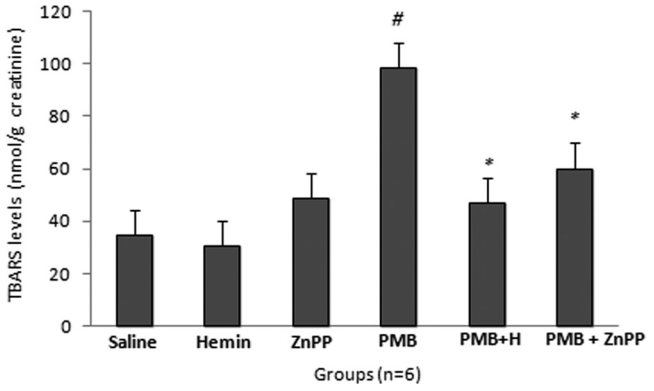

FIG 2 Mean (±SD) TBARS levels in the saline, hemin, ZnPP, PMB, PMB plus hemin, and PMB plus ZnPP groups. #, significantly different from the saline group ($P < 0.05$); *, significantly different from the PMB group ($P < 0.05$).

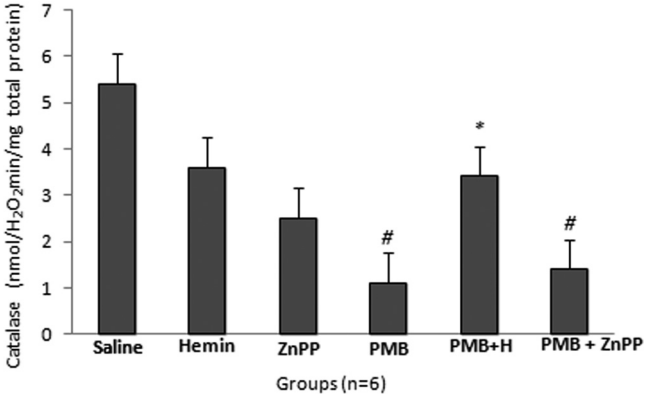

FIG 4 Mean (±SD) catalase activity in the saline, hemin, ZnPP, PMB, PMB plus hemin, and PMB plus ZnPP groups. #, significantly different from the saline group ($P < 0.05$); *, significantly different from the PMB group ($P < 0.05$).

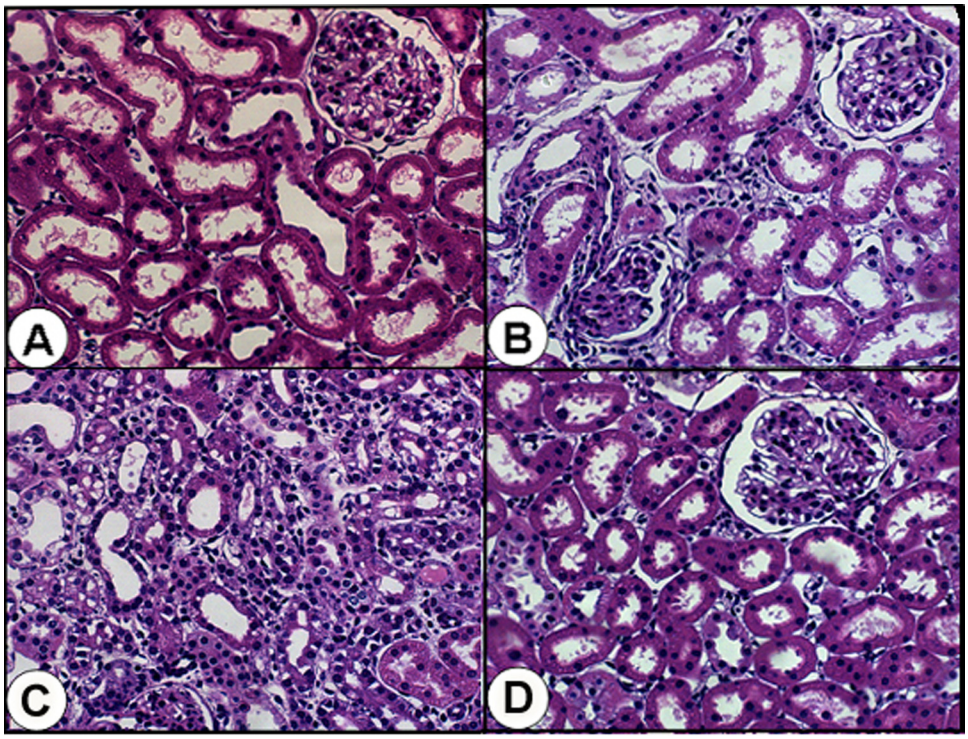

FIG 5 Histological sections of renal tissue obtained from the different groups: saline (A), PMB (B), PMB plus ZnPP (C), and PMB plus hemin (D). Magnification, ×400.

flattening of tubular cells accompanied by tubular dilatation, focal areas of a denuded basement membrane, and tubular necrosis in the renal cortex. Pretreatment with the HO-1 inducer (PMB plus hemin group) reduced these renal tubular alterations (Fig. 5D).

The results of Table 2 compare the FIA in the renal tissue in the PMB group with that in the saline group. The PMB plus ZnPP group presented an FIA that was larger than that of the saline group ($P < 0.05$) but similar to that of the PMB group.

DISCUSSION

The PMB is a peptide antibiotic that appears to have a surface detergent effect on the cell walls of Gram-negative bacteria (6, 23, 25). The detergent effect occurs when the polycationic peptide ring disintegrates the calcium and magnesium bridges and the antibiotic is inserted into the lipopolysaccharides of the outer membrane, disrupting it and causing cell lysis of the bacteria. It has also been reported that this is the mechanism by which PMB enters in human cells (6, 23).

The current reemergence of PMB is the result of the growing number of infection cases caused by multidrug-resistant microorganisms. However, its adverse effects compromise the current use.

TABLE 2 FIA in renal tissue of rats treated with saline, PMB, PMB plus hemin, and PMB plus ZnPP

Group	No. of rats	FIA (%)
Saline	4	7.5 ± 0.5
PMB	4	10.0 ± 1.0
PMB plus hemin	4	8.7 ± 0.5
PMB plus ZnPP	5	10.4 ± 1.5[a]

[a] $P < 0.05$ versus the value for saline.

Clinical studies have reported a high rate of toxicity with the PMB family, specifically nephrotoxicity and neurotoxicity (6, 37). PMB-induced nephrotoxicity is observed in 20 to 40% of seriously ill patients (9, 14, 22, 26).

The treatment with PMB for 5 days in this study caused nephrotoxicity characterized as a decrease in creatinine clearance, resulting in nonoliguric AKI in healthy adult rats. It was demonstrated that the polymyxin family can induce nephrotoxicity characterized by ATN, azotemia, or an elevated serum creatinine concentration (15, 31).

In the present study, a decrease in the activity of antioxidant enzymes and an increase in the excretion of urinary peroxides and TBARS were observed in animals treated with PMB. An oxidative imbalance probably resulted from the release of the ROS and decreased antioxidant enzyme activity. ROS increase damage to the lipid membrane, and peroxides exacerbate the catalytic reactions of cellular components. Animals treated with PMB showed a similar pattern, suggesting that this effect might be the result of direct tubular toxicity. PMB toxicity is related to the D-amino content and fatty acid component that increases permeability and disruption of the cellular membrane, resulting in cell swelling and lysis as described previously (34).

Cellular edema is one of the first signs of reversible cell damage that can be detected by light microscopy. Histological analysis of the PMB group in this study confirmed edema and diffuse inflammatory infiltration of the interstitium, flattening of tubular cells accompanied by tubular dilatation, focal areas of a denuded basement membrane, and tubular necrosis in the renal cortex. These events lead to the loss of the integrity of the mitochondrial membrane, compromising protein function

and inhibiting cell proliferation and repair (30). The release of ROS and tubular dysfunction with the establishment of acute tubular necrosis were observed in models of toxic AKI (16). Similarly, studies on colistin-induced nephrotoxicity in rats showed that the injury is mediated through oxidative stress with elevated levels of lipid peroxidation and a reduced tissue antioxidant defense, as presented in this study after treatment with PMB (32, 46, 47).

On the other hand, cells possess "protective genes" in which expression is aimed at preserving cellular structure and function in harmful environments. One of these genes encodes HO-1, an enzyme that degrades heme (2, 29).

The improvement of renal function with an increase of creatinine clearance was observed in this study in PMB animals pretreated with hemin, an inducer of HO-1. In addition, excretion of urinary peroxides and reduction in TBARS and normalization of catalase activity were found. HO-1 is induced by oxidant stress, and its robust expression provides protection against oxidative insults. The heme molecule is frequently found to be associated with proteins but might be found to be dissociated in different pathological situations. Free or intracellular heme destabilizes membranes and can catalyze the generation of free radicals by reacting with organic hydroperoxides (17). Heme degradations products, carbon monoxide, biliverdin/bilirubin, and iron/ferritin possess potent antioxidant and antiapoptotic properties (1, 4, 7, 36).

The use of ROS scavengers prevents irreversible cell damage and necrosis. Certain types of enzymatic and nonenzymatic systems inactivate oxidative reactions. These enzymes include catalases, superoxide dismutases, and glutathione peroxidase (30). In the present study, induction of HO-1 resulted in an increase of these enzymes, demonstrating its functional and structural protective effect in animals treated with PMB. Similar results were observed in studies using other antioxidant agents, such as *N*-acetylcysteine, melatonina, and ascorbic acid, in a colistin-induced nephrotoxicity model (32, 46, 47). Therefore, the present results suggest that the induction of HO-1 exerted a remarkable protective effect against the oxidative cell damage induced by PMB.

In addition to the findings regarding renal function, peroxides, TBARS, catalase and thiols, this study also demonstrates sublethal histopathological alterations in animals treated with PMB and PMB plus ZnPP, such as membrane edema, diffuse inflammatory infiltration of interstitial tissue accompanied by tubular dilatation and denuded basement membrane, and ATN in some areas of the renal cortex. These findings were ameliorated when hemin was administered during the PMB treatment.

The alterations in renal function associated with elevated levels of lipid peroxidation and reduction in the tissue antioxidant defense are characteristics of models of nephrotoxicity as mentioned above (32, 34, 46, 47). The simple increase in the levels of free radicals, such as hydrogen peroxides, triggers the onset of reversible structural injuries (30). Maintenance of the redox imbalance and the activation of lysosomal enzymes lead to an irreversible injury characterized by necrotic foci as observed in the present study.

Indeed, the present results suggest that induction of HO-1 exerts a protective effect against the degenerative cell damage induced by treatment with PMB.

In summary, this is the first study demonstrating the protective effect of HO-1 against PMB-induced nephrotoxicity. Drugs continue to be the common origin of AKI, and its prevention requires knowledge about the pathophysiological mechanisms of renal injury. The findings of this study highlight the profile of PMB-induced nephrotoxicity and reinforce the HO-1 potential effect in oxidative damage. However, future research needs to focus on methods of translating the renoprotective properties of HO-1 into clinical practice.

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